METHOD FOR ~~RECOGNIZING AND DETERMINING GNRH RECEPTORS AND THE USE OF GNRH AGONISTS AND GNRH ANTAGONISTS AND OTHER GNRH RECPETOR LIGANDS FOR THE TREATMENT WITH GNRH RECEPTORS OF~~ Diagnosis Therapy using GNRH Agonists and GNRH antagonists TUMOR$ ~~ORIGINATING IN THE BRAIN AND/OR NERVOUS SYSTEM AND/OR MENINGES AND/OR OF KAPOSI SARCOMA~~

The present invention relates to tumor diagnosis and therapy. In particular, it is directed to the diagnosis and therapy of tumors carrying GnRH receptors. The GnRH receptor is a well-known target in tumor therapy.

Post-operative treatment of prostate and mamma carcinomas with agonists of gonadotropin releasing hormone (GnRH, in the literature also referred to as luteinizing hormone releasing hormone; LH-RH) is a standard treatment; cf. Gonzalez-Barcena et al., 1994, The Prostate 24, 84-92; Emons and Schally, 1994, Human Reproduction Update 9, No. 7, 1364-1379.

Thus, in various steroid hormone (sexual hormone) dependent malignant tumors, such as mamma carcinoma, prostate carcinoma, ovarian carcinoma, and endometrial carcinoma, a double effect has been observed in clinical studies upon treatment with GnRH agonists:

1) an indirect anti-proliferative activity by uncoupling of the positive endocrine (estrogenous or androgenous) effect on tumor growth;

2) a direct anti-proliferative activity by an unknown mechanism via GnRH receptors in the tumor tissue itself; cf. Emons and Schally, 1994, Human Reproduction Update 9, 1364-1379.

The above-mentioned indirect effect due to steroid hormone dependence is known since decades for the prostate and the mamma carcinoma; cf. Gonzalez-Barcena et al., 1994, The Prostate 24, 84-92; Jonat et al., 1995, European Journal of Cancer 31A, 137-142.

The direct anti-proliferative effect of GnRH agonists and GnRH antagonists on e.g. prostate carcinomas, mamma carcinomas, and ovarian carcinomas has been confirmed by clinical studies. Some of the GnRH agonists employed in these treatments having a direct

anti-proliferative effect are known by the following trademarks of the medicaments approved in Germany: for example Zoladex®, Zoladex 10,8®, Zoladex Gyn®, Profact®-Depot, Profact pro injectione/nasal, Synarela®, Enantone Monats-Depot®, Uno-Enantone®, Enantone Gyn Monats-Depot®, Trenantone®, Suprecur®, Carcinil®, or Decapeptyl® 0,5 mg/0,1 mg, Decapeptyl® Depot, Decapeptyl® Gyn as well as Decapeptyl® Diagnostik. An example for a GnRH antagonist examined in several studies is Cetrorelix® which is not yet approved as a medicament in Germany. Treatment with Cetrorelix® bears the disadvantage that no depot preparation exists which would be active e.g. for weeks. Other examples of GnRH antagonists used experimentally are Antarelix® and Antide®, the latter also existing in one embodiment as an oral presentation form (Russel-Jones et al., 1995, Bioconjugate Chem. 6, 34-42).

Research with cell culture has revealed that GnRH receptors are present on human primary liver cell carcinomas and pancreas adenocarcinomas. In addition, the beginning of a biochemical metabolization with respect to cleavage of GnRH between tyrosine 5 and glycine 6 in rat glioma and rat neuroblastoma has been described; cf. Tao et al., 1991, Neuropeptides 20, 125-131. Ligand binding of GnRH to the GnRH receptor and its signal transduction, however, take place in a different way, namely at the eighth amino acid of GnRH, arginine, and this exclusively occurs in the case of an intact conformation of the GnRH molecule and its amino acid side chains (Naor, Z., Schacham, Sh., Harris, D., Seger, R., and Reiss, N., 1995, Signal Transduction of the Gonadotropin Releasing Hormone (GnRH) Receptor: Cross-Talk of Calcium, Protein Kinase C (PKC), and Arachnoidonic Acid. Cellular and Molecular Neurobiology, vol. 15, 527-545). In normal rat adenohypophysis where GnRH receptors reside, GnRH leads to an increased cAMP production, however, it is still unclear whether this is a direct or an indirect effect (paracrine interaction). For the function of the GnRH receptor in rat including secretion of LH as well as an increased production of LH stimulated by GnRH, the biochemical metabolization of GnRH, e.g. by means of cAMP, plays only an indirect role (Abdilnour, G., and Bourne, G.A., 1995, Adenosine 3',5'-cyclic mono-phosphate and the self-priming effect of gonadotropin-releasing hormone. Molecular and Cellular Endocrinology, 107, 1-7). Naturally, there were found GnRH receptors on human gonadotropin producing pituitary

adenomas (Alexander, J.P., and Klibanski, A., Gonadotropin-releasing Hormone Receptor mRNA Expression by Human Pituitary Tumors In Vitro, 1994, Journal of Clinical Investigation, 93, 2332-2339). To treat the indication Pubertas praecox e.g. due to the GnRH-producing hamartoma of the hypothalamus, GnRH agonists were also employed in children in a symptomatic treatment of blocking gonadotropin-producing cells in the adenohypophysis (Mahachoklertwattana, P., Kaplan, S.L., Grumbach, M.M., The Luteinizing-Hormone-Releasing Hormone-Secreting Hypothalamic Hamartoma Is a Congenital Malformation: Natural History, 1993, Journal of Clinical Endocrinology and Metabolism, 77, 118-125).

In the case of glioma and other malignant tumors of ectodermal origin, such as malignant melanoma and in particular in the case of diffusely growing tumors in the nervous system or in the case of metastases (formation of disseminations, e.g. in other organs) life expectancy is not optimistic. The same is true for Kaposi sarcoma. Glioma refers to mainly brain-localized true tumors of the central nervous system (CNS) originating in the neuroglia, i.e. from the covering and supporting tissue of the nervous system which is derived from ectoderm. These gliomas are present in various differentiation stages. Subtypes of glioma are spongioblastoma, oligodendroglioma, astrocytoma, glioblastoma, and retinoblastoma. In particular, the Glioblastoma multiforme (GBM) type of brain tumors is characterized by fast growth and extremely high recidivation rate (i.e. the percentage of patients with brain tumor recurrence following surgical macroscopic excision).

Malignant melanoma occurring in the CNS, primary or as metastasis, as well as malignant melanoma which primarily occurs in the skin and/or malignant melanoma which disseminates (metastasizes) further in the skin and/or in other body organs belong to nerve system derived tumors; cf. Shamamian et al., 1994, Cancer Immunol. Immunother. 39, 73-83; Florenes et al., 1994, Cancer Research, 54, 354-356. Malignant melanomas are derived from neuroectoderm, an embryonic layer. Burg et al., 1997, Deutsches Ärzteblatt 94, 890-895, describe a tumor growth inhibiting effect of tamoxifen for the malignant melanoma. Furthermore, glioblastoma and malignant melanoma have several tumor markers in common; cf. Shamamian et al., 1994, Cancer Immunol. Immunother. 39, 73-83; Florenes et

al., 1994, Cancer Research 54, 354-356. In the case of metastases, the prognosis is very poor; cf. Burg et al., 1997, Deutsches Ärzteblatt 94, 890-895.

Tumors originating in brain and/or nervous system and/or the meninges further comprise the neuroblastoma and the medullablastoma which in their entirety have been classified as the so-called primitive neuroectodermal tumors, abbreviated as PNET. These tumors further include the pinealoma originating in pineal body parenchyma and/or primordial germ cells in the pineal body region or the brain median. Moreover, the pineal body is associated with the origin of craniopharyngeoma (a tumor producing ß-HCG or LH-like glycoprotein, respectively; cf. Tachibana et al., 1994, J. of Neurosurgery 80, 79-84) which is considered to be an ectodermal tumor and originates in the front/upper face of the pituitary.

Both for craniopharyngeoma and meningeoma which is considered to be a benign tumor originating in arachnoidal cover cells and often adhering firmly to the inner surface of the meninges (dura mater), progesterone receptors and estrogen receptors have been described. Furthermore, androgen receptors have also been established in the case of meningeoma. In clinical studies using anti-progesterone medicaments, tumor-shrinking effects have been observed.

Up to now, the investigation of other therapies (different forms of chemotherapy, radiotherapy, etc.) in numerous clinical studies failed to provide a substantial improvement of the prognosis for tumors originating in brain and/or nervous system and/or the meninges. At the time being, the standard therapy in the case of Glioblastoma multiforme still consists of an as complete as possible surgical excision of the tumor followed by conventional radiotherapy. Under this standard therapy the statistically reported mean survival time is 9-13 months with individual variations and particularly a slightly better prognosis for younger patients having been observed.

About 30% of patients with recurrent Glioblastoma multiforme showed constant size or shrinking, respectively, of the inoperable residual brain tumor under sustained high-dosage of tamoxifen®, an anti-estrogen preparation. This tumor-inhibiting effect in glioblastoma treatment has not been attributed to its anti-estrogenic effect but to its inhibition of protein kinase C (an intracellular signal mediator); cf. Puchner et al.,

Zentralblatt für Neurochirurgie, Supplement 1996, 47. Jahrestagung Deutsche Gesellschaft für Neurochirurgie, page 44; Pollack et al., 1995, The Efficacy of Tamoxifen as an anti-proliferative Agent in vitro for Benign and Malignant Pediatric Glial Tumors, Pediatr. Neurosurgery 22, 281-288). Moreover, tamoxifen® is said to increase the sensitivity of tumor cells for platinium-containing therapeutics as well as for radiotherapy.

For Glioblastoma multiforme (WHO grade IV astrocytoma) and for glioma with a lower grade of malignancy (WHO grade II-IV astrocytoma) steroid hormone receptors have been observed in a smaller percentage of the cases (cf. Paoletti et al., 1990, J. Neurosurgery, Characteristics and biological role of steroid hormone receptors in neuroepithelial tumors, 73, 736-742). Up to now, an indirect anti-proliferative effect in the case of Glioblastoma multiforme and glioma grade II-IV has been observed in clinical studies in only about 30% of the cases by a response of the tumor to tamoxifen® (an anti-estrogen preparation)

Although recently, several relatively reasonable new developments in Glioblastoma multiforme therapy have been described, the prognosis quod vitam for patients with Glioblastoma multiforme still remains poor because of the extremely high recurrence rate despite the therapy forms tried and tested so far and because of the lack of a specific therapy and early diagnostics.

On the one hand, the invention is based on the object of providing diagnostics which detect tumors originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma already in an early stage, and on the other hand based on the object of providing a medicament for the therapy of such tumors which results in a better prognosis for all patients.

The invention relates to a method for the detection and determination of GnRH receptors on malignant cells of a tumor originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma. The invention is further directed to providing a diagnostic kit for tumors originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma. Furthermore, the invention relates to the use of GnRH agonists and GnRH antagonists and of other ligands of GnRH receptors in the preparation of a

medicament for the treatment of tumors originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma.

The direct anti-proliferative effect of GnRH agonists on brain-derived tumors, e.g. Glioblastoma multiforme, has not been described to date. It has also been unknown that GnRH receptors are present on human ectodermal tumors, such as Glioblastoma multiforme. Furthermore, it has been unknown up to now that GnRH receptors are present on Kaposi sarcoma.

The present invention contributes to the improvement in diagnosis and therapy of tumors originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma by providing a suitable target for diagnosis and therapy.

The invention is further directed to the use of diagnostic kits for the detection of GnRH receptors in immunohistological diagnostics and/or for the detection of GnRH receptor mRNA for monitoring of the therapy, aftercare for early recurrence detection during follow-up of the residual tumor still present after operation, for example a low grade glioma (G II-III WHO; cf. World Health Organization (WHO) classification of tumors of the central and peripheral nervous system, in: Kleihues et al., 1993, Histological Typing of Tumors of the Central Nervous System, Springer Verlag, Berlin-Heidelberg, New York-Tokyo) or for the detection of malignization in the sense of a Glioblastoma multiforme (G IV), and for early detection in risk groups for the screening for the presence of tumors, such as Glioblastoma multiforme, originating in brain and/or nervous system and/or the meninges.

The kit according to the present invention may be used to detect GnRH receptors on cell membranes or in body liquids, such as blood, plasma, serum, urine or liquor, tissue extracts, tissue liquids, in vitro cell culture supernatants and cell lysates. The GnRH receptor may for example be determined immunohistochemically on e.g. operatively excised tumor preparations or tissue cultures or, by means of a conventional radioimmuno assay, for example in body liquids. The diagnostic kit comprises a GnRH agonist and/or a GnRH antagonist and/or a monoclonal or polyclonal antibody against human GnRH receptors and/or one or more specific primers against GnRH receptors for example for the amplification of the cDNA of a GnRH receptor in a reverse transcriptase-polymerase chain

reaction (RT-PCR). Detection of GnRH receptors is conducted in a manner known per se using well known immunological assays, in particular using enzyme-linked immunoadsorbent assays (ELISA), or in a particular embodiment using the methods described below for the detection and determination of GnRH receptors on degenerate cells.

In a preferred embodiment, the method of the present invention for the detection and determination of GnRH receptors on degenerate cells of a tumor originating in brain and/or nervous system and/or the meninges comprises the following steps of: a) homogenizing peroperatively obtained tumor tissue, b) separating the membrane fraction, c) determination of the protein concentration in the membrane fraction of b), d) determination of the concentration of GnRH receptors in the membrane fraction of b). The present method is particularly useful for the detection and determination of GnRH receptors in tissue derived from Glioblastoma multiforme, medulloblastoma, pinealoma, neuroblastoma, craniopharyngeoma, meningeoma, chordoma, Ewing sarcoma, malignant melanoma, or Kaposi sarcoma. This method provides the possibility of tumor diagnosis.

In a particularly preferred embodiment, fresh human tumor tissue is collected for example during brain tumor surgery (peroperatively) followed by storage in liquid nitrogen. For GnRH receptor determination, the frozen tissue samples are ground and homogenized. In a centrifugation step, the samples are separated from larger tissue debris. The supernatant is again centrifuged. The resulting sediment (pellet) contains the membrane fraction which is again homogenized to obtain an as homogenous membrane suspension as possible. The membrane suspension is used in the radio receptor assay for determination of GnRH receptors. First, the protein concentration in the membrane fraction prepared is determined photometrically in a conventional and known manner e.g. using the BioRad protein assay (BioRad, Munich). Determination of the GnRH receptor concentration is performed using a known GnRH agonist, such as Buserelin® binding specifically to GnRH receptors in the membrane fraction prepared. Since the GnRH agonist has been radiolabeled, for example by ^{125}I, the concentration of bound radiolabeled GnRH agonist mirrors the concentration of GnRH receptors in the membrane fraction. The concentration of bound radiolabeled GnRH agonist is determined by means of radioactive counts per minute. Both low affinity/high

capacity and high affinity/low capacity GnRH receptor binding sites are evaluated (cf. Baumann, K., et al., 1993, Breast Cancer Research Treatment, vol. 25, page 37-46).

The invention further relates to the use of GnRH agonists or GnRH antagonists to prepare a medicament for the treatment of tumors originating in brain and/or nervous system and/or the meninges. In particular, the invention is directed to the use of GnRH agonists or GnRH antagonists to prepare a medicament for the treatment of Glioblastoma multiforme, medulloblastoma, pinealoma, neuroblastoma, craniopharyngeoma, meningeoma, chordoma, Ewing sarcoma, malignant melanoma, or Kaposi sarcoma. GnRH receptors as well as a GnRH agonist/GnRH antagonist treatment have so far been described neither for craniopharyngeoma nor for meningeoma or chordoma or Ewing sarcoma or malignant melanoma and also not for the Kaposi sarcoma. For these tumors, no blood-brain barrier exists, since they originally are extracerebral, intracranial or peripheral tumors. Therefore, the therapy according to the present invention using GnRH agonists and/or GnRH antagonists or conjugates thereof, respectively, is very advantageous. However, the blood-brain-barrier is permeable for GnRH since a two-direction-system, a bidirectional active transport of GnRH across the blood-brain-barrier exists (Barrera, C., Banks, W.A., Fasold, M.B., and Kastin, A.J., 1991, Effects of Various Reproductive Hormones on the Penetration of LHRH Across the Blood-Brain Barrier, Pharmacology, Biochemistry & Behaviour, vol. 41, 255-257). Thus the treatment by GnRH agonists/GnRH antagonists has advantages over the treatment with tamoxifen for which a blood-brain-barrier exists. For Ewing sarcoma and other peripheral forms of PNET outside of the nervous system, for malignant melanoma and for Kaposi sarcoma, the blood-brain-barrier generally does not play an essential role in the treatment with GnRH agonists/GnRH antagonists since these tumors in most of the cases arise and stay on the outside of the blood-brain-barrier.

Table I

List of GnRH agonists and GnRH antagonists which may be employed in the treatment of a tumor having GnRH receptors and originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma:

GnRH agonists:	GnRH antagonists:
Pharmacological substance name	Trade mark
Leuprorelinacetate, Leuprorelin	Cetrorelix®, Asta Medica AG, Frankfurt/Main
Triptorelinacetate, Triptorelin	Antarelix®, Asta Medica AG, Frankfurt/Main
Buserelinacetate, Buserelin	Antide®, Ares Sarono Int. SA, Lausanne, Switzerland
Goserelinacetate, Goserelin	Ramorelix®, Behringwerke, Marburg, Germany

Further examples of GnRH antagonists are:

- LHRH antagonists similar to Antide® as described in US patent 5,480,969 (Bowers et al., date of patent: Jan. 2, 1996)
- LHRH peptide derivatives as described in the UK patent GB 2 246782 B (Albert, R., et al., patent published on 16/09/1992)
- LHRH antagonists as described in US patent 5,198,533 (Schally et al., date of patent: Mar. 30, 1993)

The minimum treatment dose of the GnRH agonists in the above list corresponds to the dosage cited in the Rote Liste® for the respective GnRH agonists for other indications of use for the subcutaneous or the intramuscular administration form, respectively. For intravenous administration of GnRH agonists the minimal daily dose is employed, cf. for example Klijn et al., 1982, The Lancet, 1213-1216.

The minimum treatment dose of the GnRH antagonists Cetrorelix®, Antrarelix®, Antide®, and Ramorelix® for subcutaneous and intramuscular administration forms in the above-cited list corresponds to the dosage described in the literature and used with other

indications, cf. for example for Cetrorelix®: Gonzalez-Barcena et al., 1994, The Prostate 24, 84-92.

The minimum treatment dose of the GnRH agonists Cetrorelix®, Antrarelix®, Antide®, and Ramorelix® for the intravenous administration form in the above-cited list corresponds to the dosage which is known for other indications at the proper approval board or described in the Deutsche Pharmazeutische Stoffliste or in the literature and is administered, for example for Antide®: Fattinger et al., 1996, Am. J. Physiol. 271 (Endocrinol. Metab. 34) E775-E787. The same is true for the GnRH antagonists as described in US patent 5,480,969, UK patent GB 2 246782 B, and US patent 5,198,533.

According to the invention, GnRH agonists and/or GnRH antagonists may be employed in any suitable form. For tumors within the blood-brain-barrier, direct injection, e.g. into the circulation, intraarterially directly into the nervous system circulation or intravenously, or injection in the liquor ways or local application in the tumor bed following surgery, directly after macroscopic tumor resection, peroperatively or with Ommaya® reservoir, or another form of subcutaneous ventricular injection in the liquor ways is preferred. It is possible to use both GnRH agonists and GnRH antagonists because both bind as ligands to the GnRH receptor. Further, ligands which are specifically directed to the GnRH receptor may be used e.g. preferably human or humanized antibodies. In most cases it is preferable to ensure that the targeting agent primarily reaches tumor cells. Therefore imaging methods using the ligand with tracers are a further aspect of the invention. If the ligand is localized mainly in the tumor, the ligand may be coupled to a cytotoxic agent, such as a radioisotope or another toxic substance such as ricin A or the like. Preferred GnRH agonists are cited in the Rote Liste which is explicitly incorporated herein by reference (Rote Liste, 1997, paragraph 50, part 3, pituitary hormones, 50038 to 50056, editor ROTE LISTE® Service GmbH, Frankfurt/Main). Preferred GnRH antagonists which already have been clinically used in patients for other treatments are Cetrorelix® and Antarelix® from Asta Medica AG, Frankfurt/Main, Germany, and Antide® from Ares-Sarono Int. AG, Lausanne, Switzerland.

The above-mentioned GnRH agonists and GnRH antagonists may be administered in dosages approved for other treatments. There may also be used dosages established during

dose finding studies for the use of similar materials (substances, medicaments) such as somatostatin analogues in pituitary adenoma, glioblastoma or pancreas adenocarcinoma, or for phase II studies with GnRH analogues (agonists or antagonists) for other indications, e.g. mamma carcinoma, prostate carcinoma or ovarian carcinoma.

In a particular embodiment, the GnRH agonists or GnRH antagonists are conjugated with a gonadotropin or LH inhibitor, respectively, such as Gossypol® (cf. Flack et al., 1993, J. Endocrinol. Metab., Oral Gossypol in the Treatment of Metastatic Adrenal Cancer 76, 1019-1024; Poso, H., et al., The Lancet, 1980, 885) or with melatonin or a melatonin analogue (an agonist or antagonist) (cf. Lissoni et al., 1996, Increased Survival Time in Brain Glioblastomas by a Radioneuroendocrine Strategy with Radiotherapy plus Melatonin Compared to Radiotherapy Alone, Oncology 53, 43-46).

In the following an example for a preferred treatment protocol is described.

For the first time, the GnRH receptor concentration in cell membranes of human brain or nervous system tumor cells, i.e. the GnRH receptors on the membrane which are effective in vitro have been determined using a radio receptor assay. With the method according to the invention, the biological activity or specifically the active GnRH receptors, respectively, are determined. For this purpose, radiolabeled Buserelin®, a GnRH agonist, is used as a marker binding specifically to GnRH receptors. Based on radioactive counts of bound Buserelin® the GnRH receptor concentration may be determined. This detection has already been used for other tumors such as mamma carcinoma and the like. The method used according to the present invention measures the GnRH receptor concentration on cell membrane extracts of fresh human tumor tissue.

During peroperative resection of the tumor tissue on the one hand tissue is obtained and processed for pathological anatomical examination and on the other hand tumor tissue for GnRH receptor determination, e.g. in the manner described herein. Following pathological anatomical examination and confirmation of the histological diagnosis of a tumor originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma a prognosis may be made for a therapy success during treatment with GnRH agonists/GnRH antagonists with respect to the concentration of GnRH receptors present.

At a concentration of the GnRH receptor of more than 1000 amol/mg (= 1 fmol/mg) membrane protein the patient will be diagnosed as GnRH receptor-positive. Being not GnRH receptor-positive is no criterion for exclusion from treatment since no clinical exclusion criteria exist for GnRH agonist/GnRH antagonist treatment. The being GnRH receptor-positive of a patient is judged prognostically as a faster tendency of recidivation than that of being GnRH receptor-negative in the course of tumor growth under classical standard treatment wherein the GnRH receptor functions as a prognostic tumor marker. Also, being GnRH receptor-positive is considered to be particularly advantageous for the treatment with GnRH agonists/GnRH antagonists, and being GnRH receptor-positive or -negative provides a prognostic information of the therapy success to be expected so that GnRH receptor is a prognostic tumor marker in that treatment. The GnRH agonist/GnRH antagonist treatment is started immediately after pathological anatomical examination, e.g. postoperatively in the case of rapid section pathological diagnostics.

Following determination of the presence of GnRH receptors, a suitable ligand (GnRH agonist, GnRH antagonist or conjugates) is selected and administered to the patient from whom the tumor was derived, preferably after diagnostic imaging methods. Cf. MTT test literature: Hunter et al., 1993, Europ. J. Surg. Oncology, 242-249.

The treatment is continued as long as no complete remission has occurred. Criteria to judge the therapy effect are: A. tumor volume on MRT images and/or CAT scan images, B. recidivation-free survival, C. overall survival for initial application as well as D. Karnofsky and Spitzer indices. The dosage for administration which may be in any suitable form known to those skilled in the art is described above and below in this patent application.

The exact mechanism of action of GnRH agonists or GnRH antagonists on tumors is unknown. For the tumor types known so far having active GnRH receptors such as mamma carcinoma, prostate carcinoma and ovarian carcinoma, a locally regulatory autocrine-paracrine system has been proposed in the literature; cf. Irmer et al., 1995, Cancer Research 55, 817-822. For the tumors mentioned, anti-proliferative activities of GnRH agonists or GnRH antagonists have been described in the literature, both in vitro (Palyi et al., 1996, Cancer Detection and Prevention, 20, 146-152; Irmer et al., 1995, Cancer Research, 55,

817-822; Pati et al., 1995, Endocrinology, 136, 75-84) and in vivo or clinically, respectively; cf. Gonzalez-Barcena et al., 1994, The prostate 24, 84-92; Jonat et al., 1995, European J. of Cancer, 31A, 137-142; Emons and Schally, 1994, Human Reproduction Update 9, No. 7, 1364-1379; wherein this anti-proliferative activity goes beyond the anti-proliferative effect to be expected of reversible "chemical castration" by GnRH agonists.

For glioblastoma and glioma in a similar manner the following mechanism of action can be considered. In the literature (Constam et al., 1992, J. Immunology, 148, 1404-1410) the production of transforming growth factor ß (TGF-ß) by glioblastoma cells has been described. Growth factor TGF-ß has been described by Melcangi et al., 1995, Endocrinology, 136, 679-686, as a product of rat glia cells, i.e. normal non-tumor cells, which as a factor in vitro stimulates the natural GnRH production in hypothalamic cells. It has been postulated that GnRH produced and secreted locally by glioblastoma has a stimulating effect on the tumor growth which has also been known for TGF-ß. Also human glioblastoma cells and glioma cells, respectively, are able to secrete circulating immunosuppressive substances, mainly TGF-ß, and therefore may induce an adverse effect on cellular immune reactions. Besides a GnRH-stimulating function, the increase in TGF-ß presumably also has an immunosuppressive (defense inhibiting) effect on the cellular immunity of the patient due to which tumor growth is promoted and tumor size increases. For Glioblastoma multiforme, medulloblastoma, and malignant melanoma, this immunosuppressive phenomenon of TGF-ß has been described; cf. Stockhammer et al., 1995, J. of Neurosurgery 83, 672-681; Jennings et al., 1994, Hum. Pathol. 25, 464-475; Bizik et al., 1996, J. Cell Biochem. 62, 113-122; van Belle et al., 1996, Am. J. Pathol. 148, 1887-1894. This autocrine-paracrine growth regulating system may be reversed resulting in a decrease in tumor size. This reversion (also referred to as "negative feedback" in endocrinology) may be in principle effected by an excess of GnRH (competitive inhibition). This effect is even enhanced by using GnRH agonists or GnRH antagonists instead of GnRH. A result of this therapy is a decrease in TGF-ß production followed by a decrease in tumor size resulting therefrom. Also ß-HCG plays an immunosuppressive role. According to the invention, also the LH-ß and ß-HCG production, respectively, are inhibited by GnRH agonists or GnRH antagonists. Also, in GBM the EGF production is inhibited.

For the tumors originating in brain and/or nervous system and/or the meninges belonging to indication invention, reference is made to the World Health Organization (WHO) classification of tumors of the central nervous system which has been established in 1990 (Kleihues et al., 1993, Histological Typing of Tumors of the central nervous system, Springer Verlag, Berlin Heidelberg New York Tokyo). In addition to the tumors cited in the above-mentioned WHO classification, also malignant melanoma, Ewing sarcoma and the Kaposi sarcoma belong to the indication invention. Excluded from the indication invention are the pituitary adenoma, all metastases except Ewing sarcoma, melanoma and Kaposi sarcoma, lymphomas and hematopoietic tumors. Germ cell tumors such as chorion carcinoma are similar to malignant tumors of the placenta which are known for bearing GnRH receptors. Therefore, the germ cell tumors of the central nervous system belong to the present indication invention. The Kaposi sarcoma with multicentric occurrence in the body consists of cells of monoclonal origin (Rabkin et al., 1996, The New England Journal of medicine, 14, 988-993). It has specific antigens in common with skin neurofibroma, a tumor originating in the nervous system (Rudolph, P., et al., 1997, Am. J. Surg. Pathol. (US), 21(7), 791-800).

With respect to hormones, Kaposi sarcoma is similar to malignant placental tumors and meningeoma since Kaposi sarcoma has ß-HCG receptors as have these tumors and reacts anti-proliferatively to the administration of ß-HCG as for example does the meningeoma (Boyle-Wash et al., 1995, Effect of glycoprotein and protein hormones on human meningeoma cell proliferation in vitro, Journal of Endocrinology, 145, 155-161; Albini et al., 1997, The beta-core Fragment of human chorionic gonadotropin inhibits growth of Kaposi sarcoma-derived cells and a new immortalized Kaposi sarcoma cell line, AIDS (US), 11(6), 713-721; Gill et al., 1996, The effects of preparations of human chorionic gonadotropin on aids-related Kaposi sarcoma, The New England Journal of Medicine, 335 (17), 1261-1269). Due to the analogy to meningeoma, Kaposi sarcoma has GnRH receptors wherein the discovered autocrine connection of GnRH being known as the ß-HCG releasing hormone in placenta and placental tumors plays a role (Lin et al., 1995, J. Clin. Endocrinol. Metab. 80, 580-585). The tumors cited above in the WHO classification of central nervous system tumors as well as malignant melanoma with ß-HCG production

and/or ß-HCG receptors carry GnRH receptors. The Ewing sarcoma belongs to the group of primitive neuroectodermal tumors (PNET) and is a peripheral form of these (Grier, H.E., 1997, The Ewing Family of Tumors. Ewing sarcoma and primitive neuroectodermal tumors. Pediatric Clin. North Am. (US), 44 (4), 991-1004).

The pineal gland (Glandula pinealis) is the origin of the production of the hormone melatonin which is a GnRH receptor expression stimulating hormone in metastasizing prostate carcinoma in the case of resistance during a GnRH agonist treatment (cf. Lissoni et al., 1997, European Urology 31, 178-181) and in addition has an anti-angiogenetic activity (Regelson, W., Pierpaoli, W., 1987, Cancer Invest., 5, 379-385). GnRH agonists and GnRH antagonists have an anti-mitotic and anti-proliferative activity, respectively, by inhibiting growth factors such as epidermal growth factor (Motta et al., 1996, J. Steroid Biochem. Molec. Biol., 56, 107-11, 1996). Epidermal growth factor is also present as a mitogen and, thus, as a positive growth factor, e.g. in Glioblastoma multiforme (Rao et al., 1996, Peptides (US), 17, 179-181). Thus, a melatonin-GnRH analogue conjugate reasonably combines an anti-mitotic and anti-angiogenetic activity on tumors such as glioblastoma and induces the further expression of GnRH receptors e.g. in Glioblastoma multiforme in order to avoid resistance against GnRH agonist/GnRH antagonist treatment by GnRH receptor depletion.

According to the present invention there are provided for the first time GnRH agonists or GnRH antagonists for the preparation of a medicament for the treatment of tumors originating in brain and/or nervous system and/or the meninges and/or of Kaposi sarcoma.

According to the invention, the GnRH agonists or GnRH antagonists as well as the conjugated GnRH agonists or GnRH antagonists are used to treat tumors originating in brain and/or nervous system and/or the meninges, for example Glioblastoma multiforme. The medicaments according to the present invention may be prepared in any manner known to the skilled artisan, in particular for subcutaneous, intramuscular, intravenous, intraspinal or subdural, respectively, or intranasal application or in the form of a sustained release implantation. The medicaments according to the present invention may also be administered via a subcutaneous ventricular cytostatic reservoir being connected to the ventricle wherein

the reservoir may be replenished by injections through the skin. The GnRH agonists may be administered in the same dosage as those which are for example used in the treatment of prostate, mamma carcinoma or endometriosis; cf. e.g. Rote Liste, 1997, paragraph 50, part 3, hypothalamic hormones, 50038 to 50056, Editor ROTE LISTE® Service GmbH, Frankfurt/Main, which is included herein explicitly by reference; cf. Annex A. The minimal dose corresponds to the dose cited in the Rote Liste for the respective GnRH agonists. For example, in the case of intraspinal or subcutaneous ventricular administration via a cytostatic reservoir the minimal dosage may be lower than that cited in the Rote Liste for the respective GnRH agonists. The maximal dose corresponds to the LD_{50} value for the respective GnRH agonists. The dosage may be optionally increased or decreased following a finding of the GnRH receptor concentration obtained in a neurological manner. The frequency of application or daily dose, respectively, may also be found in the Rote Liste. Preferably, the medicaments are administered until complete remission (regression) of the tumor which may be evaluated neuroradiologically and clinically.

For subcutaneous administration, e.g. Carcinil®, Decapeptyl® 0,5 mg/0,1 mg or Uno-Enantone may be employed. As sustained release implantations for example Profact®-Depot, Zoladex®, or Enantone Monatsdepot may be administered. For intramuscular administration, e.g. Decapeptyl®-Depot, Decapeptyl®-Gyn, or Enantone-Gyn may be employed. For intranasal administration e.g. Profact®-Nasal, Suprecur®-Nasal, or Synarela®-Nasal may be used. For intravenous administration or intranasal administration, respectively, for example Profact pro injectione/-nasal may be administered in the dosage given by Klijn, J.G., and De Jong, F.H. in Klijn, J.G., and De Jong, F.H., 1982, The Lancet, 1213-1216. The GnRH antagonists may be administered in a dosage which has been for example given for Cetrorelix® in Gonzalez-Barcena et al., 1994, The Prostate 24, 84-92, or may be administered at minimum in the dosage as given for example for Antide® in Fattinger et al., 1996, Am. J. Physiol. 271 (Endocrinol. Metab. 34: E775-E787).

The following examples are intended to illustrate the invention and should not be construed as limiting the invention.

Example 1: Determination of the concentration of GnRH receptors

As an example for the determination of the concentration of GnRH receptors on cell membrane extracts of cell lines and/or cell cultures, the Decapeptyl® radio receptor assay is used with membranes (as described by Emons, G., et al., 1993, Cancer Research 53, 5439-5446). According to this protocol, the GnRH receptors are determined on a human cell line such as the human glioblastoma cell line U-87 MG or U-373MG (Pinski et al., 1994, Cancer Research 54, 5895-5901). In this test, the low affinity/high capacity as well as the high affinity/low capacity GnRH receptor binding sites are evaluated. Similar results as those described in Emons, G., et al., supra, for the cell lines EFO-21 and EFO-27 are obtained.

As another example for the determination of the concentration of GnRH receptors on cell membrane extracts of cell lines and/or cell cultures the LHRH radio receptor assay with labeled Triptorelin (Emons, G., et al., supra) is performed on a Kaposi sarcoma cell line such as the well known cell line KSY-1 or KS-SLK (Parkash et al., 1996, New England Journal of Medicine 335, 17, 1261-1269) and on a human malignant melanoma cell line such as the well known cell lines MV3 and BLM (Goldbrunner, R.H., et al., 1996, Anticancer Research 16 (6B), 3679-3687) obtaining similar results for the GnRH receptor determinations as described in Emons, G., et al., supra, for the cell lines EFO-21 and EFO-27.

Example 2: Determination of the mRNA of GnRH receptors by means of RT-PCR

As an example for the determination of GnRH receptor messenger RNA by means of RT-PCR for example RNA from the glioblastoma cell line U-87 MG or U-373MG is in a first reaction transcribed to cDNA. In a further reaction for example the 884 bp fragment of the pituitary GnRH receptor (Kakar, S., et al., Biochem. Biophys. Res. Comm., 1992, 289-295) or of the placental GnRH receptor (Leung, P.C.K., Biological Signals, 1996, 5, 63-69) or of the placental GnRH receptor gene (Lin, L., et al., J. Clinical Endocrinol. Metabolism, 1995, vol. 80, No. 2, 581-584) is amplified using specific primers in a reverse transcriptase polymerase chain reaction wherein the cDNA of a known GnRH receptor-positive cell line serves as the positive control. Then, the reaction products are visualized in a polyacrylamide (PAA) gel. On the PAA gel in lane 1 there may be seen the fragment length marker, in lane

2 a clear band of the 884 bp GnRH receptor PCR product in the MCF 7 positive control and also in the lane of the glioblastoma cell line a signal of an 884 bp product or other GnRH receptor splice variant (fragment) signals. This mRNA detection is performed similar to other GnRH receptor mRNA determinations, see for example Irmer et al., 1995, Cancer Research, 55, 817-822.

Example 3: Therapeutic in vitro study

Proliferation assay on cell cultures

A human cell line such as the well known human glioblastoma cell lines U-87MG or U-373MG (Pinski et al., supra) or a human cell line such as the well known Kaposi sarcoma cell lines KSY-1 or KS-SLK (Parkash et al., 1996, New England Journal of Medicine, 335, 17, 1261-1269) or a human cell line such as the well known human malignant melanoma cell line MV3 or BLM (Goldbrunner, R.H., et al., 1996, Anticancer Research 16 (6B), 3679-87) or a human medulloblastoma cell line such as the well known cell line Daoy or D283 MED (Stockhammer et al., 1995, J. Neurosurgery, 83, 672-681) or human meningeoma cell cultures (Boyle-Wash, E., et al., 1995, Journal of Endocrinology, 145, 155-161) are cultured as described by the above-mentioned authors for the above-mentioned cell lines and then treated as described by Emons, G., et al., 1993, supra, and Irmer, G., 1995, supra, with a concentration of the GnRH agonist Triptorelin, GnHR antagonist SB-75 (Cetrorelix®) or GnRH antagonist Ramorelix® as described therein. Similar results to those described by Emons et al., Cancer Research, 53, 1993, 539-544, and Irmer, G., et al., supra, were obtained.

Separately, the above-mentioned cell lines were also treated with an GnRH agonist, either with Goserelin (Zoladex®, Buserelin or Leuprorelin) or with a GnRH antagonist such as Antide® or Antarelix®. Similar anti-proliferative effects as those described by Pinski et al. or Irmer et al., supra, were observed.

Also separately, such cell lines were additionally treated each with one of the GnRH antagonists Cetrorelix®, Antarelix®, Antide®, and Ramorelix® or with one of the GnRH antagonists as described in US patent 5,480,969, US patent 5,198,533, or UK patent GB 2

246782 B wherein this treatment was performed similar to that reported in Emons et al., supra, for SB 75 (Cetrorelix®). A similar anti-proliferative effect occurs.

The cell lines cited above were also treated separately with monoclonal antibodies against a GnRH receptor antigen as described by Karande, A.A., et al., 1995, Mol. Cell. Endocrinol. 114 (1-2), p. 51-56. A similar anti-proliferative effect is observed for the above cell lines as has been described by Ackermann, R.C., et al., 1994, Cancer Letters, 81, 177-184, for the OVCAR-3 cell line.

Example 4: In vivo study in the model of xenotransplantation

An in vivo study with nude mice

An effect of the treatment of tumor-implanted nude mice (Pinski et al., supra) each with one of the GnRH agonists Buserelin, Triprorelin, Goserelin, and Leuprorelin and each with one of the GnRH antagonists Cetrorelix® (SB-75), Antarelix®, Antide®, and Ramorelix® on the growth of malignant gliomas U-87 MG and U-373MG was proven by us using daily doses and controls in nude mice as have been described for the determination of the efficacy of similar peptides in Pinski et al., supra. Similar growth-inhibiting effects could be observed in the above tumors by treatment with the GnRH agonists and GnRH antagonists mentioned by us.

Example 5: Phase I study

Patients with non-resectable Glioblastoma multiforme in the condition after microsurgical resection and/or after external conventional radiotherapy and/or brachytherapy or patients with a diffusely, intraaxially growing brain tumor, multifocal tumor spreading or presence of a gliomatosis cerebri, respectively, a tumor volume of more than 65 ml or a minimal tumor diameter of more than 5 cm were treated with the GnRH agonist Buserelin administered intravenously as described by Klijn, J.G.M., et al., 1982, The Lancet, May 19, 12143-1214, and also as described therein by intranasal application as a permanent medication. As the effect of the treatment a reduction in tumor volume is observed on MRT or CT images, respectively. A recidivation-free survival longer than

described for the tamoxifen treatment method of glioma (Pollack et al., 1995, Pediatr. Neurosurgery 22, 281-288) has been observed.

Example 6: Phase I study

2 Patients with inoperable, stereotactically confirmed Glioblastoma multiforme after conventional radiotherapy were treated under permanent medication with Zoladex® in the dosage and administration form as cited for metastasizing mamma carcinoma in the Rote Liste. MRT controls reveal a significant reduction in tumor volume.

Example 7: Phase II study

Patients with histologically confirmed Glioblastoma multiforme after a first operation were treated (randomized controlled) with Zoladex® as described by Jonat et al., 1995, European J. Cancer, 137-142. Following radiotherapy, they are assigned to two groups. One group is treated with Zoladex® and one group without Zoladex® (or with Cetrorelix® and without Cetrorelix®, or with Antide® and without Antide®, or with Decapeptyl® or without Decapeptyl® etc.). The effects are similar to the metastasized perimenopausal mamma carcinoma. The percentage showing an actual significant therapy effect is evaluated according to the criteria of tumor volume, recidivation-free survival, overall survival following initial application and Karnofsky and Spitzer indices in a clinical neurological examination and under consideration of the other examination criteria (Sposto, R., et al., 1989, J. Neurooncology, 7, 165-177, and Kirby, S., et al., 1995, J. Natl. Cancer Institute, 87, 1884-1888, 1995). In MRT and/or CAT scan, a significantly higher reduction in tumor volume or significantly longer recidivation-free survival and significantly longer overall survival following initial application, respectively, than in the control group not treated with Zoladex® have been observed.

By using a method of gene therapy well-known to the skilled artisan retroviruses and antisense GnRH receptor vectors are stably transfected into glioma cells, and an anti-proliferative effect is observed.

Example 8: Collection of glioma tissue

During brain tumor operations (peroperatively) fresh human tumor tissue was collected dry in a small sterile dish without addition of medium and immediately transferred into a sterile standard plastic tube. The tube was sealed air-tight and after about 15 minutes shock-frozen in a Dewar container (Union Carbide Cryogenic Equipment 35HC, ref. No. 103-139-T5) containing liquid nitrogen. The tissue samples were stored in liquid nitrogen for about 2 months until GnRH receptor determination.

Example 9: Tissue preparation

The frozen tissue samples were cleaned from residual blood and fat and cut into pieces of about 2 x 2 x 2 mm using a scalpel. The tissue samples were homogenized for 1 minute at maximum output in a Dismembrator II (B. Braun, Melsungen). The homogenized tissue was resuspended in 1000 µl of cold buffer 1 (10 mM tris-(hydroxymethyl)-aminomethane, pH 7.4, 4°C) and mixed as homogenous as possible. In a first centrifugation step (800 x g, 10 minutes, 4°C) the sample was separated from larger tissue debris. The supernatant was again centrifuged (10.000 x g, 45 minutes, 4°C). The supernatant of the second centrifugation step was discarded, and the pellet containing the membrane fraction was resuspended in 1000 µl of cold buffer 1and homogenized using a Polytron homogenizer three times for 4 seconds each to obtain an as homogenous membrane suspension as possible. To this membrane fraction, 1000 µl of cold buffer 1 were added. This suspension was used in the determination of GnRH receptors in the radio receptor assay.

Example 10: Determination of the protein concentration

The BioRad reagent was diluted 1:5 with distilled water. 3.5 ml of this reagent were mixed with 50 µl of the membrane fraction prepared and incubated for 5 minutes. Photometric measurement of the protein concentration was carried out as a double determination at a lambda of 595 nm in a well-known manner. A human albumin protein standard which is correspondingly used for the measurement serves as the protein standard.

Example 11: The radio receptor assay

The determination of the concentration of GnRH receptors was carried out in the membrane fraction of the tissue prepared as described above. The radio receptor assay comprised two different samples each of which is determined in fourfold: a) samples containing the prepared membrane fraction, and b) control samples.

a) 300 µl buffer 2 (10 mM tris-(hydroxymethyl)-aminomethane, pH 7.4, 0.1% bovine serum albumin) and 100 µl of tracer (^{125}I-Buserelin, 80.000 cpm/100µl) were added to 100 µl of membrane fraction.

b) For the controls, 250 µl buffer 2, 100 µl of tracer, 100 µl of membrane fraction and 50 µl GnRH analogue (10^{-5} M Buserelin) are mixed.

The individual samples were well mixed and then incubated for 90 minutes at 4°C. The radio receptor assay was stopped by addition of 500 µl of bovine gamma globulin solution (0.1% bovine gamma globulin, 0.15 M NaCl). Subsequently, 1000 µl of a 25% PEG-6000, 0.15 M NaCl solution were added.

The samples were again mixed until homogenous and incubated for 20 min at 4°C. Separation of the PEG-hormone receptor complexes was performed via a centrifugation step (1.600 x g, 30 minutes, 4°C) during which the complexes due to their higher mass form the pellet. The supernatant is removed carefully using a Pasteur pipette. The number of counts per minute serving as a basis for evaluation of the GnRH receptor content was then determined in a Gamma counter (Berthold).

Example 12: Examination of the radio receptor assay

Generally, several tissue samples were used in an experimental approach. To exclude a systematic error in the case of a negative result of all samples in one assay, a standard sample from bovine pituitary tissue was examined in each of the assays in parallel to the tumor tissues. Thus, the detection of GnRH receptors in bovine pituitary tissues served as a positive control. The pituitary tissue was prepared similar to the tumor tissues and the membrane fraction was purified in a similar manner.

Example 13: Evaluation of the GnRH receptor content

The evaluation of the GnRH receptor content (fmol/mg of membrane protein) was carried out on the basis of the counts per minute (cpm), the specific binding, the amount of protein used, and the specific activity of the radiolabeled ligand.

The specific binding (B_{spec}) is calculated from the difference of the mean value of the fourfold determination of total binding (B_0) and the mean value of the fourfold determination of unspecific binding (NSB).

The amount of protein used is determined photometrically as described above under 3.

Data of the analogue ^{125}I-Buserelin:

MG:	1253 g/mole
Specific Activity:	1470 mCi/mg
Activity of ^{125}I-Buserelin solution	20 µCi/ml

- 1470 mCi/mg ^{125}I-Buserelin = 54.4×10^9 Bq/mg
- 1ml of ^{125}I-Buserelin solution includes 13.61×10^{-9} g ^{125}I-Buserelin with 7.4×10^6 Bq
- 13.61×10^{-9} g/ml ^{125}I-Buserelin = 10.9×10^{-12} mole ^{125}I-Buserelin, 54.4×10^9 Bq = 44.4×10^7 cpm.
- 10.90×10^{-12} mole ^{125}I-Buserelin = 44.4×10^7 cpm
- 1000 cpm correspond to 0.247×10^{-15} mole ^{125}I-Buserelin.

For the calculation of the GnRH receptor concentration (fmol/mg of membrane protein) from the cpm values measured also the amount of protein used and the disintegration factor has to be considered. Thus, the equation for the calculation of the GnRH receptor content is the following:

$$\frac{0.247 \times 10^{-15} \text{ mole } ^{125}\text{I-Buserelin}}{\text{disintegration factor} \times \text{amount of protein}} = 1000 \text{ cpm}$$

Table II

Determination of the GnRH receptor concentration

The results of the GnRH receptor determination using the radio receptor assay according to the invention of tissue samples of several patients are listed.

Histological samples	ER fmol/mg prot	PgR Fmol/mg prot	GnRH rec. atomol/mg prot	Finding
	10	20	1000	negative
	10-20	20-30	1000-3000	weakly positive
	20	30	3000-5000	positive
	50	100	5000	strongly positive
Chordoma	1	1	708	
GBM	1	2	2478	
GBM	1	1	895	
GBM	1	1	1111	
G II Glioma	1	1	3635	
Meningeoma	1	74	1	
Adenocarcinoma	1	1	1	
GBM	1	1	7357	
Fibrillary				
G II Astrocytoma	1	1	1	
Meningeoma	1	177	7444	
Meningeoma	1	550	1588	
GBM	1	1	4466	
Additional values:				
-Chordoma	1	1	1117	weakly positive
-Intraspinal meningeoma	3	7	1640	weakly positive
-Brain metastasis of plate epithelium carcinoma of the lung	1	1	200	negative
-Normal brain tissue	4	1	460	negative

ER=estrogen receptor, PgR=progesterone receptor, GnRH rec.=GnRH receptor

Example 14: Proliferation assay using the human malignant melanoma cell line MV3

The human melanoma cell line MV3 was cultured (in long-term culture in RPMI medium (Gibco Co.) with 1% Penstrep and 10% of heat-inactivated fetal calf serum). The proliferation assay was carried out with 6 x 10^2 cells per well in 96 well plates. First, the cells were removed from the culture flask with a 0.02 mM solution and then washed in standard PBS solution. Following centrifugation for 10 minutes (1200 g) the supernatant was discarded and the pellet resuspended in 1 ml medium. An aliquot of 20 µl of the cells was diluted with trypan blue to obtain an 1:20 dilution. Trypan blue stains the necrotic cells. Then counting was performed in a Neubauer counting chamber. Evaluation was performed by daily determination of 4 values starting at day 0 and multiplying the mean values of the cell counts x 10^4 x dilution factor 20 to obtain the cell count. During 5 days, the measurement was performed 4 x daily in a Biomec spectrophotometer.

The method for determination of tumor cell proliferation is described in Lü, H.Q., et al., 1996, Journal of Cancer Research and Clinical Oncology, 122, 335-342.

The cell line was treated with (Gly-OH10)-LHRH, the LHRH hormone (Figure 3) (Sigma Chemical Co., No. L8008) or Triptorelin, an LHRH agonist (Figure 2) (Sigma Chemical Co., No. L9761) or Antide, a LHRH antagonist (Figure 1) (Sigma Chemical Co., No. A8802).

In the concentrations of 10^{-4} M, 10^{-5} M, and 10^{-6} M using medium as a negative control from day 4 on the following results were obtained:

Referring to Fig. 1: For Antide (GnRH antagonist) a clear inhibition of proliferation is seen in the high concentrations of 10^{-4} M and 10^{-5} M of 15% and 35%, respectively, (similar as described by Emons et al., 1993, supra, but with later onset as compared to the ovarian carcinoma cell lines used therein in which an anti-proliferative effect of the antagonists in one of the two cell lines occurred from day 1 on). At a concentration of 10^{-6} M no inhibition of the proliferation was observed but a stimulation of the growth of 40%. This paradox in vitro effect of GnRH antagonists is similar to that described in Limonta et al., 1993, J. Clin. Endocrinol. Metab., 76, 839-845, for prostate carcinomas with GnRH receptors. A similar in vitro effect for relatively low concentrations is also known for tamoxifen in the MCF-7 mamma carcinoma cell line (Zänker, K., et al., 1995).

For Triptorelin (GnRH agonist) (see Figure 2) an inhibition of the proliferation of 15% was observed from day 4 on at the concentrations mentioned. In Emons et al., 1993, supra, this has been observed already starting from day 1 for both ovarian carcinoma cell lines under a Triptorelin treatment of 10^{-5} M, and 40% inhibition was observed on day 6.

These findings indicate the presence of a direct anti-proliferative effect of Antide and Triptorelin on malignant melanoma. It has also been proven that GnRH receptors are present on the human malignant melanoma cell line MV 3 since binding of a non-ligand to the tumor cells can be excluded.

The graphs of Figures 1-3 prove that malignant melanoma MV3 is a LHRH homone-dependent tumor.

Thus, also in vitro the LHRH hormone functions as a positive growth factor. The function of LHRH hormone produced in an autocrine manner is inhibited by Antide and Triptorelin.